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Maternal Stress and Effects of Prenatal Air Pollution on Offspring Mental Health Outcomes in Mice

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Abbreviations

CORT= corticosterone

DEP= diesel exhaust particles

E18= embryonic day 18

ELISA= enzyme-linked-immunosorbent assay

HIPP= hippocampus

HYP= hypothalamus

IL-1β= interleukin-1 beta

IL-10= interleukin-10

LG= licking and grooming behavior

MACS= magnetic-activated cell sorting

NR= nest restriction

P0= postnatal day 0

PCX= parietal cortex

PFC= prefrontal cortex

qRT-PCR= quantitative real-time polymerase chain reaction

SES= socioeconomic status

TLR4= toll-like receptor 4

VEH= vehicle

ABSTRACT

Background: Low socioeconomic status is consistently associated with reduced physical and mental health, but the mechanisms remain unclear. Increased levels of urban air pollutants interacting with parental stress have been proposed to explain health disparities in respiratory disease, but the impact of such interactions on mental health is unknown.

Objectives: We aimed to determine whether combined prenatal air pollution exposure and stress during pregnancy act synergistically on offspring to induce a neuroinflammatory response and subsequent neurocognitive disorders in adulthood.

Methods: Mouse dams were intermittently exposed via oropharyngeal aspiration to diesel exhaust particles (DEP; 50 μg x 6 doses) or vehicle throughout gestation, combined with standard housing or nest material restriction (NR; a novel model of maternal stress) during the last third of gestation.

Results: Adult [postnatal day (P)60] offspring of dams that experienced both stressors (DEP/NR) displayed increased anxiety, but only male offspring of this group had impaired cognition. Furthermore, maternal DEP exposure increased proinflammatory IL-1β levels within the brains of adult males but not females, and maternal DEP and NR both decreased anti-inflammatory IL-10 in male, but not female, brains. Similarly, only DEP/NR males showed increased expression of the innate immune recognition gene, toll-like receptor (TLR)4, and its downstream effector, caspase-1.

Conclusions: These results show that maternal stress during late gestation increases the susceptibility of offspring—particularly males—to the deleterious effects of prenatal air pollutant exposure, which may be due to a synergism of these factors acting on innate immune recognition genes and downstream neuroinflammatory cascades within the developing brain.

INTRODUCTION

Although low socioeconomic status (SES) has been repeatedly associated with a higher rate of chronic health problems and mental disorders (Adler and Rehkopf 2008; Reijneveld and Schene 1998), explicit characterization of the factors that underlie this phenomenon remains elusive (Evans and Kantrowitz 2002). A growing body of research suggests that maternal well-being during pregnancy is a crucial determinant of lifelong physical and mental health of the off-spring (Case et al. 2005; Hackman et al. 2010; Susser et al. 1999). Notably, expectant mothers living in low SES conditions experience the greatest burden of toxins and pollutants (Evans and Kantrowitz 2002), along with fewer resources and high psychological stress (Seguin et al. 1995). Chemical toxins such as lead exposure are well known to adversely affect brain development (Needleman et al. 1990; Weiss and Landrigan 2000). However, "social toxins", such as violence, poverty, and other factors that generate psychological stress in low SES parents and children, have only recently begun to gain recognition as risk factors that can also alter the trajectory of brain development (Wright 2009). For example, a recent study noted an association between air pollution and asthma only in children that were also living with a chronic stressor (e.g. domestic violence) (Clougherty et al. 2007). Similarly, parental stress can increase the effect of *in utero* toxin exposures (i.e., tobacco smoke) on childhood asthma risk (Shankardass et al. 2009). In such cases, stress may increase vulnerability, permitting a toxin to initiate significant injury to physiological systems when it would have been insufficient to do so in isolation. Importantly, these synergistic effects of stress and pollutants are possible because they likely act on common biological systems, such as innate immune pathways (Frank et al. 2007; Levesque et al. 2011) within the developing nervous system.

Air pollution, one of the most relevant and pervasive environmental toxins in the modern world, is a particularly important threat to child health and is increasingly associated with neurodevelopmental disorders such as autism (Volk et al. 2013). Mechanistic studies have revealed that diesel exhaust, a major component of air pollution, markedly activates microglia, the resident immune cells of the brain, in adult rats (Levesque et al. 2011). Furthermore, our research has demonstrated that maternal exposure to diesel exhaust causes long-term increases in microglial antigen expression in the brains of adult offspring (Bolton et al. 2012). Notably, prior stress enhances proinflammatory cytokine expression and associated neural damage following an immune challenge in adult rats (De Pablos et al. 2006), most likely by sensitizing microglia (Frank et al. 2011). Because cytokines are important for normal brain development and adult function (Boulanger 2009; Yirmiya and Goshen 2011), significant perturbations in their expression may have enduring consequences for lifelong mental health (Dantzer et al. 2008).

Thus, we hypothesized that the addition of maternal stress to the impact of prenatal air pollution exposure would act synergistically in offspring to impair mental health outcomes, compared with the effects of either exposure alone. In order to test this hypothesis, we combined our animal model of prenatal diesel exhaust exposure (Auten et al. 2012) with an adaptation of a novel model of maternal resource deprivation (nest restriction; NR) (Rice et al. 2008). Pregnant mice were exposed to intermittent aspiration of vehicle or diesel exhaust particles (DEP) throughout gestation, and either lived in normal housing or had reduced nesting materials during the last third of pregnancy.

METHODS

Animals

We obtained adult male and female C57BL/6 mice from Charles River Laboratories (Raleigh, NC) and time-mated them in four separate cohorts (see Supplemental Material). After confirmation of successful mating [vaginal plug, considered to be embryonic day (E)0], we paired females in individually ventilated cages with specialized bedding (AlphaDri; Shepherd Specialty Papers, Milford, NJ) and *ad libitum* access to food (PicoLab Mouse Diet 5058, Lab-Diet, Philadelphia, PA) and filtered water. All mice used in this study were treated humanely and with regard for alleviation of suffering, and experiments were conducted with protocols approved by the Duke University Animal Care and Use Committee.

Prenatal Stressors

DEP exposures

Beginning on E2, we lightly anesthetized time-mated females with 2% isoflurane for ~1 min and treated them with DEP via oropharyngeal aspiration (Auten et al. 2012) (see Supplemental Material). Females received 50 μg DEP suspended in 50 μl vehicle (DEP group) or vehicle alone (VEH group) every 3 days E2-E17 for a total of 6 doses, as a model of intermittent exposure. This dose and route of delivery induces maternal lung inflammation (e.g. white blood cell infiltration) comparable to levels observed following intermittent maternal inhalation of diesel exhaust at environmentally relevant concentrations (Auten et al. 2012). Moreover, both routes of delivery result in similar levels of particle deposition within the lung (Foster et al. 2001).

Maternal resource deprivation

We adapted a model of postnatal nest restriction (Rice et al. 2008) by applying it to the prenatal period, and used the degree of restriction that produced minimal phenotypic changes with NR alone. Beginning on E14 following DEP exposures, we singly housed half of the VEH- and DEP-treated dams in clean cages with a thin layer of bedding under an elevated fine-gauge aluminum mesh platform (mesh dimensions 0.4 cm × 0.9 cm; McNichols Co., Tampa, FL) and provided them with 2/3 of one square of felt-like nesting material (~1.9 g; NR group). The remaining dams we singly housed in clean cages with bedding and one full square of nesting material (~2.8 g; Control group). On E19, we placed NR dams in clean cages with normal bedding and one full square of nesting material, and from that point forward, treated them identically to Control dams. This design resulted in 4 groups of dams: VEH/Control (n=8), DEP/Control (n=10), VEH/NR (n=8), and DEP/NR (n=10).

Neonatal Outcomes and Maternal Behavior

Birth weights

All animals included in this study were allowed to deliver spontaneously on gestational days 19-20 [defined as postnatal day (P)0], and offspring were not cross-fostered. We weighed pups (sex not determined) on P1 in order to acquire a litter average for birth weight (n=8-10 litters/group from 3 cohorts). Four weeks later, we weaned offspring into clean cages of 2-5 same-sex siblings, and provided them with *ad libitum* access to standard chow and filtered water.

Neonatal corticosterone (CORT) measurement

On P1, we randomly selected one pup from each litter (males: n=5-8/group; females: n=3-7/group from 2 cohorts), performed rapid decapitation, and collected trunk blood to obtain a

measure of basal circulating CORT levels soon after birth. We assessed total serum CORT concentrations using an enzyme-linked-immunosorbent assay (ELISA; Enzo Life Sciences, Inc., Ann Arbor, MI) (Bilbo et al. 2007).

Maternal behavior assessment

In order to characterize the effect of prenatal stressors on maternal care, we observed dams (n=3-7/group from 2 cohorts) twice daily with their litters P2-P9 to measure time spent on the nest, nursing, and licking and grooming (LG) their pups (Myers et al. 1989) (see Supplemental Material).

Fetal Brain Cytokine Analysis

In order to assess the fetal brain cytokine response to DEP and/or NR, we euthanized a separate cohort of identically treated dams (n=2-3/group) at E18. Thus, we obtained fetuses by hysterotomy under sodium pentobarbital anesthesia (250 mg/kg i.p.), placed them on ice, and decapitated them. We snap-froze whole fetal brains and stored them at -80°C until processing. We also collected tail snips for later genotyping to determine the sex of each fetus. From these tail snips, we extracted genomic DNA (Kouduka et al. 2006), and subsequently assessed SRY PCR products (Koopman et al. 1991) (see Supplemental Material, Table S1).

We used ELISAs (R&D Systems, Minneapolis, MN) to measure interleukin (IL)-1β, a proinflammatory cytokine, and IL-10, an anti-inflammatory cytokine, in fetal brain homogenates normalized to total protein (200 μg/well) and lipid-depleted (n=7-8/sex/group; see Supplemental Material). We selected these cytokines due to their important role in microglial function, brain development, and behavior (Deverman and Patterson 2009; Williamson et al. 2011; Yirmiya and Goshen 2011).

P30 Neuroimmune Gene Expression

To determine the long-term effect of prenatal stressors on genes critical for innate immune recognition and the subsequent cytokine response in the brain, we assessed male (n=8-13/group; 2-4 litters represented/group from 1 cohort) and female offspring (n=5/group; 2-4 litters represented/group from 1 cohort) at P30. Thus, we deeply anesthetized animals with 430 mg/kg ketamine and 65 mg/kg xyalzine i.p. and transcardially perfused them with ice-cold saline for 2 min to clear brains of blood. Afterwards, we extracted the brains, removed the cerebellum and hindbrain, and cut the remaining forebrain sagittally in half. We snap-froze half-brains and stored them at -80°C until quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Williamson et al. 2011) (see Supplemental Material).

Behavioral Procedures

In order to assess behavioral outcomes as a result of prenatal stressors, we tested young adult (P60-P90) male and female offspring (n=7-9 animals/sex/group, 2-4 litters represented/group from 1 cohort) with the sequence of behavioral tests listed below, with 1 week between tests. We tested males and females separately and performed all testing during the animals' dark cycle between 10 AM and 4 PM. Throughout testing, we also monitored females' estrous cycles.

Contextual and auditory cue fear conditioning

Our methods for assessing memory have been described in detail for rats (Williamson et al. 2011), but we implemented slight modifications for mice in these studies (see Supplemental Material). Briefly, we trained mice to associate a foot shock with a specific tone and a context, and tested their memory of these associations 48 hr later by assessing freezing behavior (the prototypical rodent fear response). We used contextual fear conditioning to assess hippocampal-

dependent memory, which we have previously found to be uniquely vulnerable to early-life insults (Williamson et al. 2011) in comparison with auditory cue fear conditioning, which does not require the hippocampus (Phillips and LeDoux 1992).

Elevated zero-maze

In order to assess anxiety-like behavior, we adapted a widely used method for rodents that measures time spent in the closed vs. open arms of a circular maze (Shepherd et al. 1994) (see Supplemental Material). Immediately following the test (<5 min), we collected a small blood sample (~100 μl) from the facial vein of each mouse in a separate room in order to determine CORT levels induced by the test.

Forced swim test

In order to assess depressive-like behavior, we adapted a commonly used method for mice by measuring time spent immobile in a container of water (Castagné et al. 2010) (see Supplemental Material).

Adult Brain Cytokine Analysis

In order to determine the enduring effects of prenatal stressors on brain cytokines, and their potential role in observed behavioral changes, we assessed cytokine protein levels in the brains of adult offspring 10 days following behavioral testing. Thus, we anesthetized and perfused all offspring (n=7-9/group/sex) as described above, after which we extracted brains and dissected them on ice into hypothalamus (HYP), prefrontal cortex (PFC), hippocampus (HIPP), and adjacent parietal cortex (PCX). We selected these regions for their known roles in the cognitive and affective behaviors we assessed. In order to obtain enough total protein for analysis, we pooled the dissected regions for each animal, snap-froze them, and stored them at -80°C until

processing. We performed ELISA protein analyses of IL-1β and IL-10 as described above (see Supplemental Material).

Microglial Isolation and Gene Expression Analysis

In order to determine the cellular source of the measured cytokines, we anesthetized and perfused a separate group of adult (~P60) behaviorally naïve offspring (n=5/sex/group; 2-3 litters represented/group from 1 cohort, including each of the 4 maternal treatment groups) as described above. Again, we pooled dissected HYP, PFC, HIPP, and PCX from each animal in order to obtain enough cells for later analysis. We isolated microglia by magnetic-activated cell sorting (MACS) (Williamson et al. 2011), using Miltenyi's Neural Tissue Dissociation Kit (P), anti-myelin microbeads, and anti-CD11b (an established marker for microglia) microbeads (Miltenyi Biotec, Inc., Auburn, CA). Afterwards, we washed cells in sterile PBS and stored them at -80°C until qRT-PCR was performed (Williamson et al. 2011) (see Supplemental Material).

Data Analysis

We analyzed all data with SPSS statistical software (IBM, Armonk, NY). Due to heterogenous variance, we log-transformed neonatal CORT data. Furthermore, for ELISA analyses, samples that had undetectable levels of IL-1 β or IL-10 were assigned a value of half the lowest detectable value in the assay (Thompson et al. 2012). We used three-way (Sex × DEP × NR) ANOVAs to analyze all data, except for the PCR data from CD11b+/- isolated cells, for which we used four-way (Sex × DEP × NR × cell population) ANOVAs. We followed up interactions with sex with separate 2-way (DEP × NR) ANOVAs for males and females to identify sex-specific effects, and subsequently followed up significant DEP × NR interactions within each sex with post hoc comparisons (Tukey's HSD) to identify group differences, assuming significance

for $p<0.05$. All reported p values are two-tailed, except for the correlations between behavioral measures and cytokine measures from adult brains, which utilized one-tailed p values because we had clear *a priori* hypotheses based on the apparent correspondence between observed group differences in the two measures. Finally, litter effects were controlled for by using multiple litters per treatment group, and can also be excluded due to the sex-specific effects observed in males and females from the same litters.

RESULTS

Neonatal Outcomes and Maternal Behavior

Previous research has shown that *postnatal* NR results in more frequent dam departures from the nest, decreased pup weights and increased plasma CORT at P9 (Rice et al. 2008). In this adaptation of the model, *prenatal* NR decreased birth weights [main effect of NR, $F(1,32)=12.09$, $p<0.005$], regardless of prenatal DEP exposure, but weights normalized by P8 (Figure 1A). Importantly, there were no significant differences in litter size or composition due to either environmental stressor (see Supplemental Material, Table S2). In addition to the effect on birth weight, prenatal NR also increased P1 serum CORT in male pups [main effect of NR, $F(1,20)=7.89$, $p<0.05$], but not females [Sex×NR interaction, $F(1,35)=2.78$, $p<0.05$; Figure 1B]. However, unlike postnatal NR, prenatal NR did not affect maternal behavior during P2-P9, a critical period for changes in maternal care to affect brain development (Avishai-Eliner et al. 2001). Specifically, there were no significant group differences in percent time dams spent on the nest (Figure 1C), nursing (Figure 1D), or licking and grooming (LG) their pups (Figure 1E). Furthermore, prenatal stressors did not have any enduring effects on maternal anxiety-like behavior when tested in the elevated zero-maze 60 days post-partum (Figure 1F).

Fetal Brain Cytokine Analysis

The proinflammatory cytokine, IL-1β, was not detectable in most of the E18 brain samples, and there were no significant group differences due to sex or prenatal stressors (Figure 2A). On the other hand, the anti-inflammatory cytokine, IL-10, was detectable in a greater proportion of samples, and there was a significant Sex×DEP interaction [$F(1,55)=4.24$, $p<0.05$]. Follow-up tests revealed that males tended to downregulate IL-10 in response to DEP exposure [trend for main effect of DEP, $F(1,27)=2.43$, $p=0.1$], whereas females tended to upregulate IL-10 in response to DEP [trend for main effect of DEP, $F(1,28)=2.395$, $p=0.1$; Figure 2B].

P30 Neuroimmune Gene Expression

A preliminary mouse inflammatory response 84-gene PCR array (SABiosciences/Qiagen), performed on P30 male brains, identified TLR4 as the only gene that exhibited a synergistic effect of DEP and NR (data not shown). Notably, TLR4 is an innate immune receptor critical for the response to both environmental toxins and stress (Arbour et al. 2000; Caso et al. 2008). We replicated this result with single-analyte qRT-PCR, finding a significant DEP×NR interaction [$F(1,34)=16.79$, $p<0.001$] for P30 male brains. Specifically, the DEP/NR group had significantly higher TLR4 expression than both the DEP/Control and VEH/NR groups ($p<0.05$; Figure 3A). Interestingly, there were no significant differences among P30 females [Sex×DEP×NR interaction, $F(1,51)=9.18$, $p<0.005$]. In addition, expression of caspase-1, a downstream effector molecule and key enzyme for IL-1β production (Black et al. 1988), exhibited a similar pattern to TLR4 expression. Post hoc tests revealed that DEP/NR male brains had significantly higher expression of caspase-1 than all other groups [DEP×NR interaction, $F(1,37)=14.66$, $p<0.001$; post hoc, $p<0.01$], whereas there were no significant differences among females [Sex×DEP×NR interaction, $F(1,54)=6.45$, $p<0.05$; Figure 3B].

Memory

Following contextual and auditory cue fear conditioning of adult offspring, DEP/NR males froze significantly less than all other groups when assessed for contextual fear recall [DEP×NR interaction, $F(1,29)=5.15$, $p<0.05$; post hoc, $p<0.05$]. However, DEP/NR females exhibited no such hippocampal-dependent memory impairment [trend for Sex×DEP×NR interaction, $F(1,57)=2.23$, $p=0.1$; Figure 4A]. Importantly, this male-specific deficit was not due to generalized hyperactivity or an inability of the DEP/NR males to freeze, as there were no significant differences in freezing in a novel context or freezing to the auditory cue (Figure 4A). Furthermore, the females' stage of estrous cycle did not significantly affect their behavior in any of the tests (data not shown).

Anxiety- and Depressive-like Behavior

In the elevated zero-maze, both adult male and female DEP/NR offspring spent more time in the closed arms, indicative of increased anxiety [DEP×NR interaction with sexes combined, $F(1,55)=7.350$, $p<0.01$; Figure 4B]. Post hoc tests revealed that DEP/NR males were significantly more anxious than both VEH/Control and DEP/Control males ($p<0.05$), and there was a trend for DEP/NR males to be more anxious than VEH/NR males ($p=0.07$), which did not significantly differ from VEH/Control males. Furthermore, DEP/NR females were more anxious than DEP/Control females ($p<0.05$), whereas VEH/control and VEH/NR females did not differ from each other. However, this increase in anxiety in the DEP/NR animals was not associated with increased serum CORT, as NR males exhibited a slight decrease in CORT immediately following the test, regardless of prenatal DEP exposure [main effect of NR, $F(1,20)=5.286$, $p<0.05$], and females displayed no significant differences (Supplemental Material, Figure S1A).

Finally, neither males nor females displayed any significant group differences in the forced swim test (Supplemental Material, Figure S1B).

Adult Brain Cytokine Analysis

Ten days following behavioral testing, male brains from DEP groups exhibited increased levels of IL-1β protein [main effect of DEP, $F(1,22)=7.84$, $p<0.01$], whereas there were no group differences among female brains [trend for Sex×NR interaction, $F(1,45)=2.49$, $p=0.1$; Figure 5A]. On the other hand, maternal DEP exposure and NR both caused decreased levels of IL-10 protein, in an additive fashion, in male brains [main effect of DEP, $F(1,22)=12.13$, $p<0.005$; main effect of NR, $F(1,22)=6.89$, $p<0.05$], whereas female brains again did not exhibit any significant group differences due to prenatal stressors [Sex×DEP interaction, $F(1,45)=7.10$, $p<0.05$; Figure 5B]. Overall, DEP/NR males exhibited a greater proinflammatory bias (IL-1β/IL-10 ratio) (de Wit et al. 2010) than DEP/NR females [Sex×DEP interaction, $F(1,39)=4.26$, $p<0.05$; Sex×NR interaction, $F(1,39)=4.53$, $p<0.05$; post hoc, $p=0.08$; Figure 5C].

It was striking to us that the group differences in brain cytokine measures seemed to parallel the behavioral differences we had observed in the same animals, and indeed, further analyses revealed that the brain levels of IL-1β and IL-10 correlated significantly with the behavioral measures of memory and anxiety, though in a divergent manner in males and females. Males exhibited a significant negative correlation between IL-1β and contextual fear memory [$r(26)=-0.33$, $p<0.05$], such that higher levels of proinflammatory IL-1β were associated with decreased freezing in the fear context, whereas females showed a trend for a positive correlation [$r(27)=0.27$, $p=0.09$; Figure 5D]. IL-1β was also positively correlated with anxiety-like behavior in males [$r(24)=0.37$, $p<0.05$], such that higher levels of IL-1β were associated with increased

time spent in the dark arm of the elevated zero-maze, whereas in females IL-1β was negatively correlated with anxiety-like behavior [$r(26)=-0.46$, $p<0.01$; Figure 5E]. On the other hand, anti-inflammatory IL-10 exhibited no significant correlations with memory in males or females (Figure 5F), but it was negatively correlated with anxiety-like behavior in males [$r(25)=-0.42$, $p<0.05$] and not females (Figure 5G).

Gene Expression of Isolated CD11b+ and CD11b- Cells

To assess the purity of the isolated cells, we confirmed that CD11b mRNA was expressed only in CD11b+ cells, as expected [main effect of cell population, $F(1,64)=71.68$, $p<0.001$; Supplemental Material, Figure S2A]. In contrast, CD11b- cells expressed ~20-fold higher GFAP and BDNF mRNA than CD11b+ cells (all *p's*<0.001; Supplemental Material, Figure S2B,C), indicating that this cell population (~80% of total cells) contains astrocytes and neurons. Isolated CD11b+ cells (microglia) had ~170-fold higher levels of mRNA for IL-1β and ~80-fold higher levels for IL-10 than CD11b- cells (all *p's*<0.001; Supplemental Material, Figure S2D, E). In addition, CD11b+ cells expressed ~40-fold more caspase-1 and ~20-fold more TLR4 than CD11b- cells (all *p's*<0.001; Supplemental Material, Figure S2F,G). However, there were no significant group differences in gene expression due to sex or prenatal stressors.

DISCUSSION

We report for the first time that maternal stress during late pregnancy exacerbates the impact of air pollution *in utero* on mental health outcomes in adult offspring, which are associated with alterations in neuroinflammatory tone. The adult males of dams exposed to combined stressors exhibit both striking memory deficits and anxiety, whereas females display only slightly increased anxiety. Importantly, the impact of prenatal events on offspring behavior is always

complex, and may involve changes in several physiological pathways, as well as potential alterations in maternal-offspring interactions after birth—both of which can profoundly modify neural development (Caldji et al. 1998). Maternal stress reduced birth weight and increased corticosterone at birth in males, independent of maternal exposure to diesel exhaust particles. However, maternal care was not significantly altered, and no changes in maternal anxiety were observed as a consequence of prenatal treatment. Furthermore, no corticosterone differences were detected in adulthood that could explain behavioral changes in the combined stressor offspring. Instead, the alterations in brain cytokines, occurring in a sexually dimorphic manner, may underlie distinct behavioral phenotypes in adult male and female offspring. An optimal balance of central proinflammatory cytokines, such as IL-1β, is critical for mental health, including mood regulation and hippocampal-dependent learning and memory (Yirmiya and Goshen 2011). In contrast, high levels of anti-inflammatory IL-10 are protective against behavioral changes due to microglial-driven neuroinflammation (Schwarz et al. 2011). Therefore, the diminished IL-10, in combination with increased IL-1β, in DEP/NR males could underlie their relative vulnerability to cognitive impairments and mood dysregulation, in comparison to DEP/NR females, which do not exhibit such a proinflammatory bias. The underlying mechanism of this sex difference warrants further exploration. Importantly, there is a male bias in the prevalence of neurodevelopmental disorders, including learning disabilities (Flannery et al. 2000) and autism (Stone et al. 2004), in addition to gender differences in childhood outcomes following maternal stress during pregnancy (Cao et al. 2012; Fang et al. 2011).

Our data from isolated CD11b+ and CD11b- cells demonstrate that microglia, not neurons or astrocytes, are the primary source of the measured cytokines in the brain, suggesting that they are

a target of "programming" by the prenatal stressors. Microglia begin to colonize the rodent brain around E9-10 (Ginhoux et al. 2010) and critically shape several aspects of normal brain development. Importantly, microglia largely remain in an activated, amoeboid state until the early postnatal period (Ling and Wong 2004), which we have demonstrated makes them especially sensitive to long-term functional changes by perinatal inflammatory events (Bilbo and Schwarz 2009; Williamson et al. 2011). However, we did not detect any significant differences in gene expression of cytokines in isolated adult microglia due to prenatal stressors, despite the clear differences in cytokine protein. This discrepancy may stem from the fact that the animals used for protein analysis underwent behavioral testing, whereas the animals used for CD11b isolation and gene expression analysis were behaviorally naïve. It is possible that behavioral testing may serve as a sufficient stressor to elicit relatively long-term increases in cytokine levels (i.e., enduring until tissue collection) in the brains of the DEP/NR animals, which would not be observed at baseline. Alternatively, there may be additional regulatory mechanisms at work. For instance, the selective increase in caspase-1 expression in DEP/NR males supports a role for the inflammasome, which is critical for the cleavage of proIL-1β into its mature form (Latz 2010), although we did not examine this directly. Importantly, TLR4 signaling is required for the activation of the inflammasome (Bauernfeind et al. 2009). Furthermore, DEP and maternal stress-induced signals may converge on microglia via TLR4, which we show is predominantly expressed by microglia, consistent with our previous findings in rats (Schwarz and Bilbo 2013), and is exaggerated in the brains of DEP/NR males. TLR4 is an important innate immune receptor that recognizes pathogen-associated molecular patterns (e.g., LPS), but also endogenous danger-associated molecular patterns released in response to cellular distress (e.g., DEP-induced hyaluronan or HMGB1) (Bianchi 2007). Notably, glucocorticoids may upregulate TLRs on

microglia, augmenting subsequent neuroinflammatory responses (Frank et al. 2010; Garate et al. 2013). Thus, TLR4 upregulation may occur to a greater extent in males than females as a result of the significant increase in serum corticosterone in males born to stressed dams.

Although the alterations in IL-1β we observed in adulthood were not present in the fetal brain, we did observe a significant sex difference in the fetal brain IL-10 response to maternal DEP exposure. Specifically, DEP males exhibited a downregulation of IL-10, whereas DEP females displayed an upregulation of this anti-inflammatory cytokine, which may have been protective against the impairment of neurodevelopment by prenatal stressors. In addition to brain cytokines, the placenta has been increasingly implicated as a key player in brain development, as well as in the fetal response to prenatal insults (Hsiao and Patterson 2012), and warrants further exploration in our model. Thus, our working hypothesis is that maternal stress-induced changes in TLR4 signaling enhance the effects of a chemical exposure such as DEP, likely involving the maternal-placental-fetal interface (Auten et al. 2009; Auten et al. 2012), and ultimately converging onto microglia within the fetal brain, resulting in the long-term alteration of brain function and behavior.

CONCLUSIONS

In closing, we have demonstrated for the first time that maternal psychological stress induced by resource deprivation during late pregnancy increases the vulnerability of murine offspring, particularly males, to *in utero* air pollutant exposure. Furthermore, it is clear that developmental exposure to maternal stress and air pollution, similar to other environmental compounds such as pesticides and LPS (Ling et al. 2004) can have a long-lasting impact on microglial function and neuroinflammation. Future studies aimed at elucidating the complex interactions of psychosocial

and chemical stressors will be critical for informing environmental and public health policy and identifying effective interventions.

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FIGURE LEGENDS

Figure 1. Effects of prenatal DEP and NR on neonatal outcomes and maternal behavior. (*A*) Prenatal NR decreased P1 pup weights, but weights normalized by P8. (*B*) Prenatal NR increased P1 serum CORT in male, but not female pups. (*C-E*) Neither prenatal DEP nor NR altered the percent of time dams spent On Nest, Nursing, or LG their pups during P2-P9. (*F*) Prenatal DEP and NR did not have enduring effects on maternal anxiety-like behavior 60 days post-partum. Data are mean of n=8-10/group ± SEM for pup weights, mean of n=3-8/group ± SEM for P1 CORT, and mean of n=3-7/group ± SEM for maternal behavior (**p<0.05, NR vs. Control groups).

Figure 2. Effects of prenatal DEP and NR on the fetal brain cytokine response. (*A*) No significant group differences were detected in IL-1β protein levels in the E18 brain. (*B)* Maternal DEP exposure elicited a sexually dimorphic IL-10 response in the fetal brain, such that male brains exhibited a downregulation of IL-10, whereas female brains exhibited an upregulation. Data are mean of n=7-8/group ± SEM ($^{\#}p$=0.1 DEP vs. VEH groups, and significant Sex×DEP interaction, p<0.05).

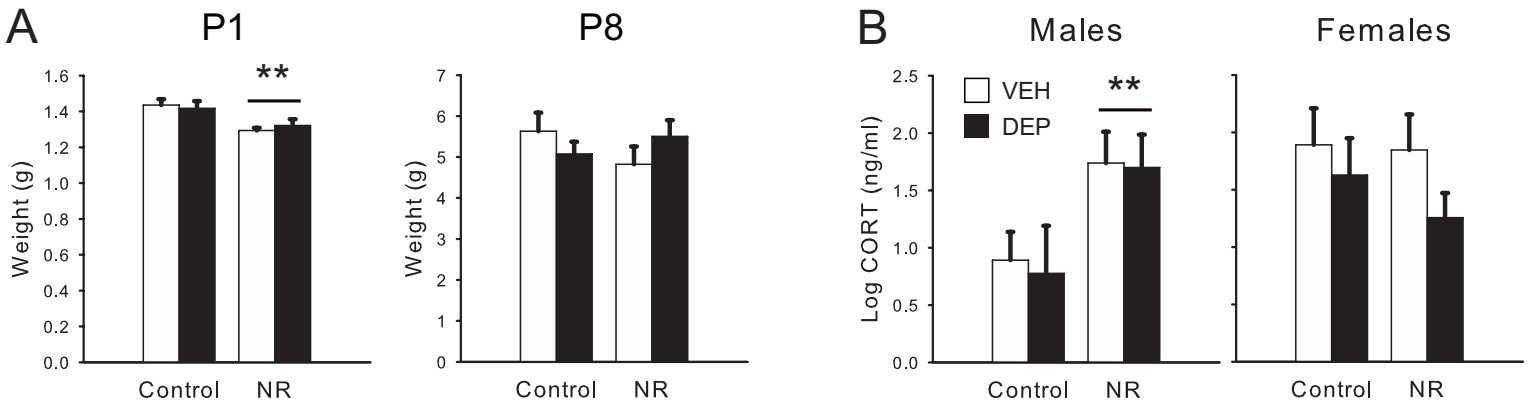
Figure 3. Effects of prenatal DEP and NR on P30 neuroimmune gene expression. The brains of DEP/NR males displayed increased TLR4 (*A*) and caspase-1 (*B*) expression, whereas DEP/NR female brains did not. Data are mean of n=5-13/group ± SEM (**p<0.05 vs. DEP/Control and VEH/NR; *p <0.05 vs. all other groups).

Figure 4. Effects of prenatal DEP and NR on cognitive and affective behavior of adult offspring. (*A*) DEP/NR males displayed decreased freezing to the fear context, which is indicative of a hippocampal-dependent memory deficit, whereas DEP/NR females did not. (*B*) Both male and

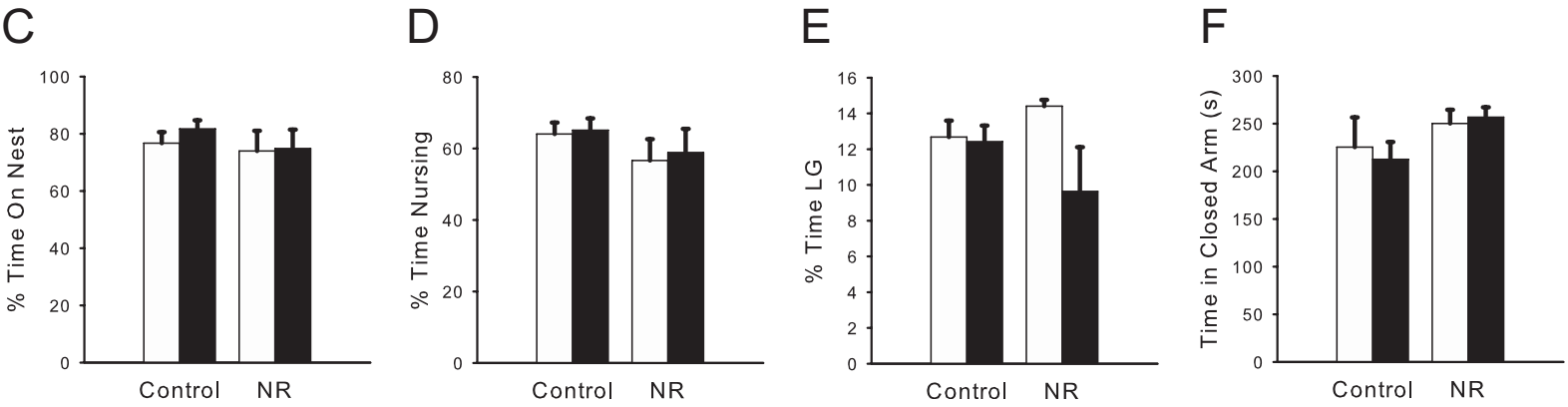
female DEP/NR offspring spent more time in the closed arm, which is indicative of anxiety-like behavior. Data are mean of n=7-9/group ± SEM (*p<0.05 vs. all other groups; **p<0.05 vs. DEP/Control and VEH/Control, p=0.07 vs. VEH/NR; $^{\#\#}p$<0.05 vs. DEP/Control).

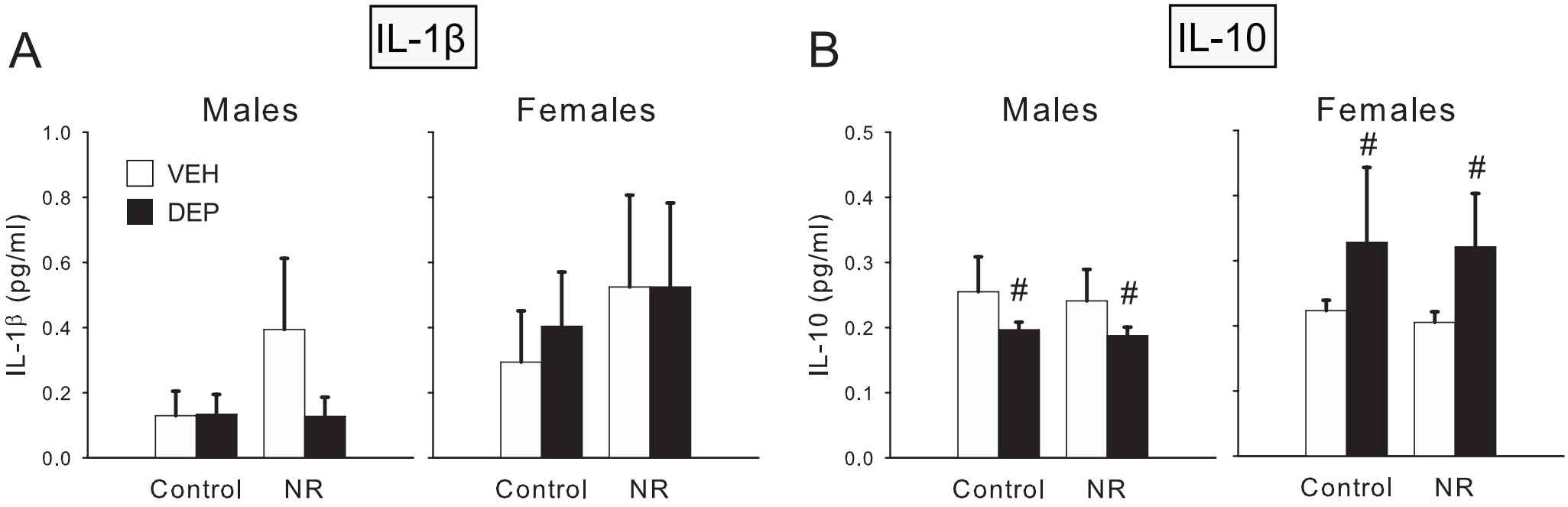
Figure 5. Effects of prenatal DEP and NR on adult brain cytokine levels. (*A*) Male offspring exhibited a significant increase in brain IL-1β due to prenatal DEP exposure, whereas females did not. (*B*) Male offspring exhibited a significant decrease in brain IL-10 in response to both DEP and NR, whereas females did not. (*C*) Overall, DEP/NR males exhibited a greater proinflammatory bias (IL-1β/IL-10 ratio) than DEP/NR females. Data for *A*-*C* are mean of n=5-8/group ± SEM ($^{\#}p$<0.05 DEP vs. VEH groups; **p<0.05 NR vs. Control groups; $^{\#\#}p$=0.08, DEP/NR males vs. DEP/NR females, and significant Sex×DEP and Sex×NR interactions, p<0.05). (*D*-*E*) Brain IL-1β was significantly correlated with memory and anxiety measures in both males and females, though in opposite directions. (*F*) Brain IL-10 was not correlated with memory performance in males or females. (*G*) Brain IL-10 was negatively correlated with anxiety-like behavior in males, but not in females. Data for *D*-*G* are correlated measures for individual animals from the whole cohort (n=24-27 total/sex).

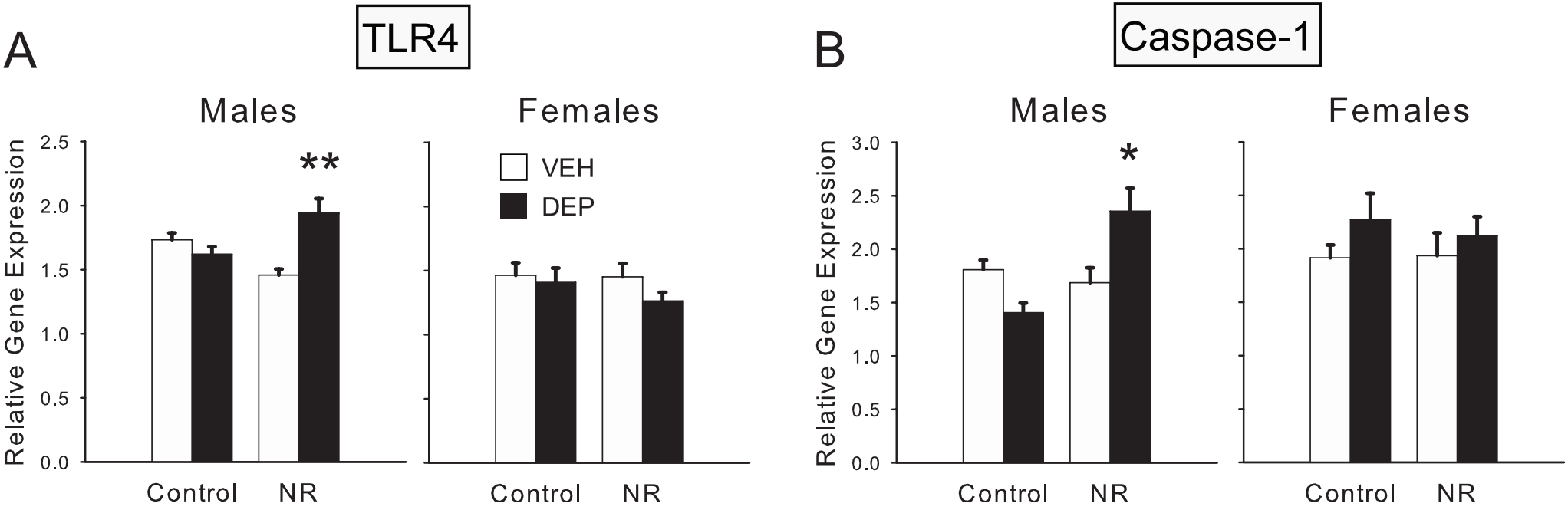
Neonatal Outcomes

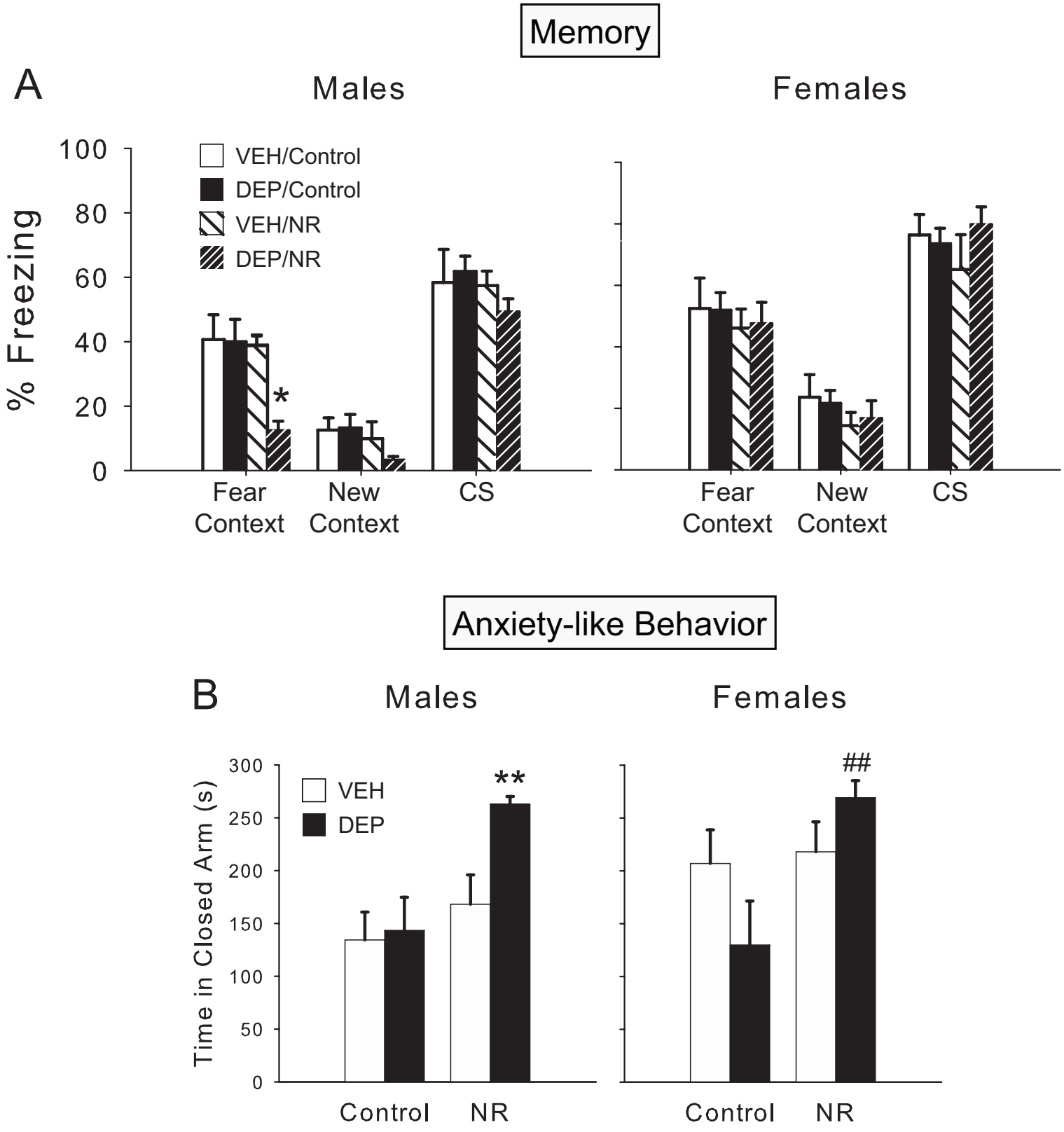


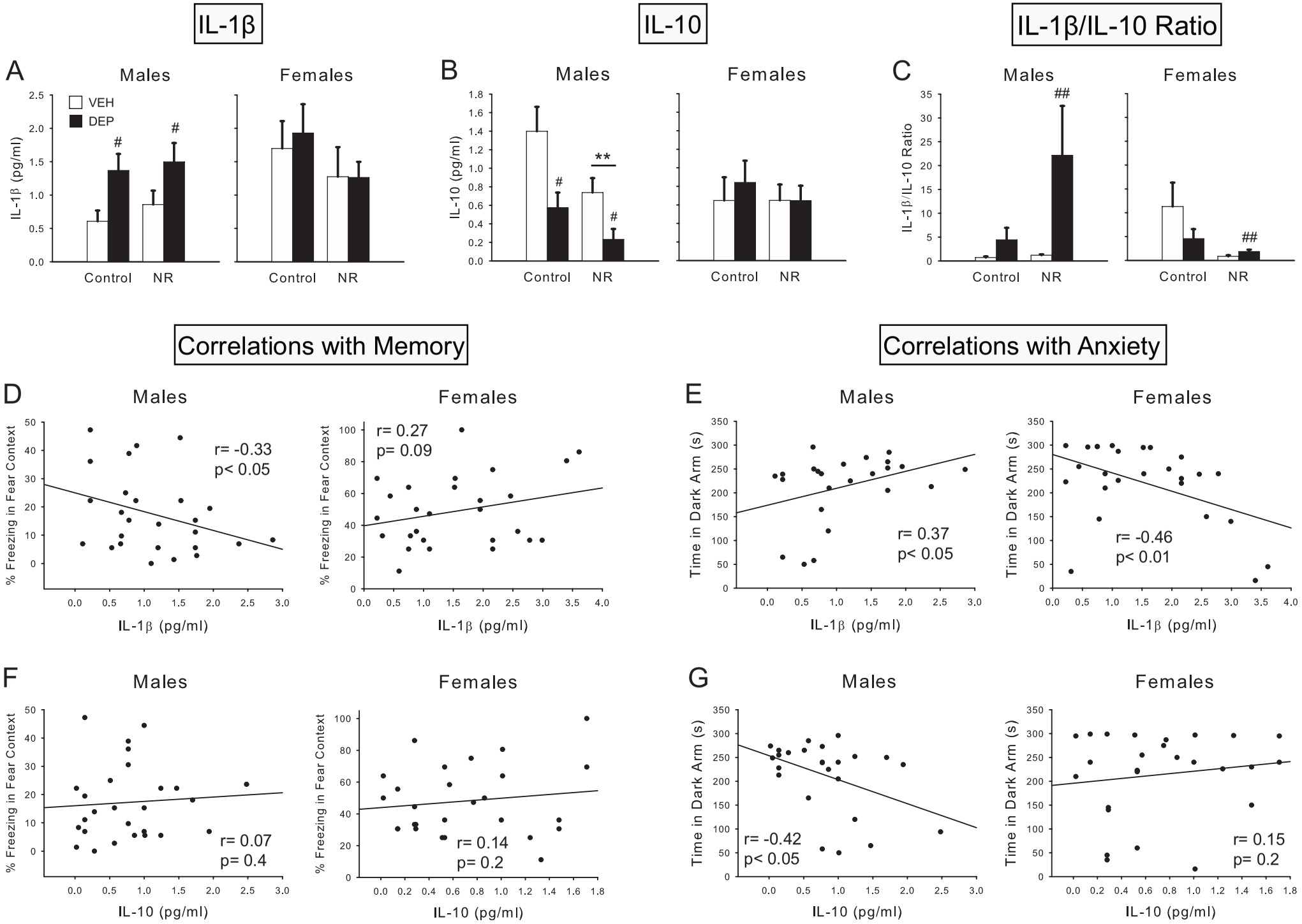
Maternal Behavior




A
IL-1β
Males
Females
VEH
DEP
IL-1β (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Control
NR
Control
NR
B
IL-10
Males
Females
IL-10 (pg/ml)
0.5
0.4
0.3
0.2
0.1
0.0
#
#
#
#
Control
NR
Control
NR


A
TLR4
Males
Females
VEH
DEP
Relative Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
NR
Control
NR
B
Caspase-1
Males
Females
Relative Gene Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
NR
Control
NR


Memory
A
Males
Females
% Freezing
100
80
60
40
20
0
VEH/Control
DEP/Control
VEH/NR
DEP/NR
*
Fear Context
New Context
CS
Fear Context
New Context
CS
Anxiety-like Behavior
B
Males
Females
Time in Closed Arm (s)
300
250
200
150
100
50
0
VEH
DEP
**
##
Control
NR
Control
NR


IL-1β
IL-10
IL-1β/IL-10 Ratio
A
Males
Females
VEH
DEP
IL-1β (pg/ml)
Control
NR
B
IL-10 (pg/ml)
C
IL-1β/IL-10 Ratio
Correlations with Memory
Correlations with Anxiety
D
% Freezing in Fear Context
r= -0.33
p< 0.05
r= 0.27
p= 0.09
E
Time in Dark Arm (s)
r= 0.37
p< 0.05
r= -0.46
p< 0.01
F
IL-10 (pg/ml)
r= 0.07
p= 0.4
r= 0.14
p= 0.2
G
r= -0.42
p< 0.05
r= 0.15
p= 0.2